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(71) Applicant: MARTEK CORPORATION [US/US]; 6480 Dobbin Road, Columbia, MD 21045 (US).

(72) Inventor: KYLE, David, J. ; 1801 Narbeth Road, Catonsville, MD 21228 (US).

(74) Agents: ERNST, Barbara, G. et al.; Rothwell, Figg, Ernst & Kurz, 555 Thirteenth Street, N.W., Suite 701 East, Washington, DC 20004 (US).

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(54) Title: MICROBIAL OIL MIXTURES AND USES THEREOF

(57) Abstract

The present invention relates to compositions including blends of microbial oils, methods of using such compositions, particularly as supplements for infant formula, and methods of increasing the amount of long chain polyunsaturated fatty acids in infant formula.

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MICROBIAL OIL MIXTURES AND USES THEREOF

This invention relates to blends or mixtures of polyunsaturated fatty acid-containing microbial oils and to uses thereof. In a specific preferred embodiment, this invention concerns the use of such oils as an additive or supplement for human diets, for example, as an additive to infant formula.

It long has been known that long chain polyunsaturated fatty acids (PUFAs) are essential to the human diet, particularly during periods of rapid tissue growth. Sanders et al, Am. J. Clin. Nutr., 31:805-813 (1978). Certain of these long chain acids, such as arachidonic acid (ARA), cannot be synthesized de novo in humans. Only by metabolizing linoleic acid (LOA), which is converted to gamma linolenic acid (GLA), and then to ARA can the human body produce ARA. LOA, in turn, is an essential acid which can only be obtained from dietary sources. Additionally, the presence of eicosapentaenoic acid (EPA) in the diet inhibits the metabolic conversion of LOA to ARA. Carlson, et al., INFORM, 1:306 (1990). ARA and docosahexaneoic acid (DHA) are critical elements of muscle, organ and vascular tissues.

Infancy is the most significant period of rapid growth in a human's life. An infant can increase its body weight by three times or more during its first y ar of life. Accordingly, it is critical that the infant receive ad quate amounts of PUFAs to insure

proper structural and organ development. Human breast milk contains high levels of PUFAs in which the ratio of ARA to EPA is typically about 20:1. However, many women choose not to breast feed their infants for either part or all of the first year of the infant's life.

As recognized by Clandinin et al., U.S. Patent 4,670,285, incorporated herein by reference, available infant formulas are deficient in long chain (C_{20} and C_{22}) PUFAs. Clandinin et al. disclose an infant formula prepared from a blend of vegetable oil and egg yolk lipid and/or fish oil which can provide a total fat composition comparable to that of human breast milk. A preferable composition comprises from 75 to 95 parts by weight egg yolk and 5 to 25 parts vegetable oil. This composition is the entire lipid content of the infant formula and it is not economical to prepare. Additionally, the infant formula disclosed by Clandinin et al. results in an EPA level which is 16 times higher than the level of EPA in human breast milk and an ARA level which is only one quarter that of breast milk.

DE 3603000A1 (Milupa) discloses a computer profile of a highly polyunsaturated acid fat mixture and discusses the use of such a mixture to produce infant formulas. Sources of the fatty acids are listed as certain types of macroalgae (i.e. seaweed), fish oil, organ fats from beef and pork, and highly refined egg yolk oil. In addition to DHA from fish oil, a potential source of DHA and ARA is said to be macroalgae, but only of the seaweed types. There is no suggestion to use microbes of any type, much less microbial oil.

Methods of producing microbial oils are disclosed in the following references, each of which is

incorporated herein by r ference. Co-pending U.S. Patent Application 07/496,572, filed March 21, 1990, discloses the production of eicosapentaneoic acid-containing single cell oils (EPASCO). Co-pending U.S. Patent Application 07/479,135, filed February 13, 1990, discloses the production of docosahexaneoic acid-containing single cell oil (DHASCO). Co-pending U.S. Patent Application 07/645,454 relates to the production of arachidonic acid-containing single cell oil (ARASCO). EP322,227 also discloses a microbial oil production system. None of these references teach the use of blends containing unmodified microbial oils as a dietary supplement, or the use of a blend of microbial oils as an additive to existing infant formula to provide that formula with a long chain PUFA composition similar to breast milk.

Accordingly, it is an object of the present invention to provide a PUFA-enriched additive, the composition of which when added to commercial infant formula will provide desired long chain PUFAs in amounts comparable to the amounts of those PUFAs found in human breast milk.

It is an additional object of the present invention to provide an economical method of producing the above-described composition.

These, and other, objects are satisfied by the present invention as described herein.

Summary of the Invention

This invention relates to the use of microbial oils which contain long chain polyunsaturated fatty acids. Additionally, in various embodiments, fish oil and/or vegetable oils can be blended with such microbial oils to form desir d compositions. The

compositions can be used as dietary supplements, particularly as additives for infant formula, as well as for pharmaceutical and cosmetic applications.

The invention also relates to economically viable processes for altering the long chain polyunsaturated fatty acid composition of infant formula and/or baby food. Preferably, the altered composition resembles that of human breast milk.

Detailed Description of the Preferred Embodiment of the Invention

Broadly stated, the present invention concerns blends, or mixtures, containing unmodified microbial oils. As used herein, "unmodified" means not chemically or covalently altered. It will be understood that throughout this specification references to "microbial oil" or "oil" mean, unless otherwise specifically stated, unmodified oil. "Microbial oils" or "single cell oils" are those oils naturally produced by microorganisms during their lifespan. Such oils can be high in long chain PUFAs. The applicant has discovered that certain of these oils, when blended with other microbial oils, fish oils, vegetable oils, or any combination thereof, can produce a composition useful for dietary, pharmaceutical or cosmetic purposes.

Various microbial oils, for example, can be obtained by, for example, the processes disclosed in above-referenced U.S. Patent Applications 07/496,572, 07/479,135, EP322,227 (Yamada et al., Suntory) or U.S. Patent Application 07/645,454. The disclosure of each of these references is specifically incorporated by reference herein.

It is to be understood that the present invention encompasses the use of a single-microbial oil containing at least two desirable PUFAs, such as ARA and DHA. The oils specifically disclosed and utilized herein, however, each contain a single desirable PUFA.

Any non-toxic, PUFA-containing microbial oil can be used in the present invention. The most preferred microbial oils are those rich in an omega-3 or omega-6 PUFA, especially DHA, GLA or ARA. These PUFAs typically are missing from, or are inadequately provided in, dietary supplements such as infant formulas or baby food. "Infant formula" as used herein means an enteral nutritional product which can be substituted for human breast milk in feeding infants and typically is composed of a desired percentage of fat mixed with desired percentages of carbohydrates and proteins in an aqueous solution. Frequently micronutrients, such as trace metals and vitamins or other desired additives are present. Examples of such micronutrients and other additives are disclosed by Clandinin et al., U.S. Patent No. 4,670,285, the disclosure of which is incorporated herein by reference.

In the present invention, types of oils from different microbes can be mixed together to obtain a desired composition. Alternatively, or additionally, PUFA-containing microbial oil can be blended with fish oil, vegetable oil or a mixture of both tc obtain a desired composition.

An objective in mixing the oils is to obtain an additive which will provide an infant formula with a desired omega-3 and omega-6 PUFA composition similar tc that found in breast milk. While the proportion of the desired fatty acids in a microbial oil can vary, this

proportion can easily be determined and the amount of oil adjusted to provide the desired amount of PUFA. Similarly, the percentage of desired PUFA in fish oil or vegetable oils can easily be determined and the amount of the oil to be added can be adjusted as necessary to achieve the desired results.

"Fish oils" are those oils obtained from fish. Such oils typically contain DHA in amounts ranging from 3% to about 20%. Typically, however, fish oils also contain EPA which depresses the production of ARA in the body. The addition of a microbial oil containing high levels of ARA to fish oil-containing compositions substantially overcomes that problem.

"Vegetable oil" includes all those oils from plants which contain long chain PUFAs. Typically, vegetable oils do not contain long chain PUFAs (PUFAs at least 20 carbons long), which is why animal organ oils are usually characterized as the source of PUFAs. Thus, vegetarians, especially vegetarian mothers, can have a diet containing inadequate amounts of PUFAs. Vegetable oils known to contain PUFAs may contain GLA. GLA is a C18:3 omega-6 PUFA. Such oils include black currant seed oil, borage oil and primrose oil. While GLA is the metabolic precursor to ARA, the process of conversion is very slow, requiring the participation of the enzyme Δ6-desaturase. This enzyme is present in humans in very low levels. Burre, et al., Lipids, 25:354-356 (1990). Thus, it would be preferable to provide the body with ARA rather than its precursor, GLA.

Methods for isolating vegetable oils are known to those of skill in the art and do not comprise a part of the present invention. Additionally, certain fungi

produce PUFA-containing oils. For example, *Mucor* species produce a GLA-containing oil.

DHASCO, defined herein as docosahexaneoic acid-containing single cell oil, can be obtained, for example, from *Crypthecodinium cohnii* as disclosed in above-referenced U.S. application 07/479,135. DHA is a C22:6 omega-3 long chain PUFA.

EPASCO, defined herein as eicosapentaneoic acid-containing single cell oil, can be obtained, for example, from *Nitzschia alba* as disclosed in above-referenced U.S. application 07/496,572. EPA is a C20:5 omega-3 long chain PUFA.

ARASCO, defined herein as arachidonic acid-containing single cell oil, can be obtained from species such as *Pythium insidiosum*, or *Mortierella alpina*, as described in U.S. application 07/645,454. ARA is a C20:4 omega-6 long chain PUFA.

Another aspect of the invention discloses a process for supplementing or altering the composition of commercially available infant formula so as to provide them with a PUFA composition more nearly like that typically contained in human breast milk. "Typical" as used herein refers to the average amounts of PUFAs measured. One of the advantages of the present invention is that, if desired, a nursing mother choosing to switch to formula can have her breast milk analyzed for PUFA content. Then, an additive for a commercially available formula which will supply comparable amounts of PUFAs can be specifically designed. Long chain PUFA-containing microbial oils from at least two microorganisms can be obtained and blended together to provide the desired composition. The blend then can be add d to an infant formula. Preferably, an amount of the blend effective to provide

an amount of the d sired PUFAs substantially similar to that found in human breast milk will be provided.

Typically, human breast milk contains from about 0.5 to 0.6% of its fatty acid content as ARA, from about 0.15 to about 0.36% of its fatty acid content as DHA and from about 0.03 to about 0.13% of its fatty acid content as EPA. Thus, a preferred ratio of ARA:DHA:EPA is from about 5:1:1 to about 20:10:1 respectively. Amounts of oils providing approximately these ratios of PUFAs can be determined without undue experimentation by those of skill in the art.

In a preferred embodiment, the microbial oils include ARASCO and DHASCO and EPASCO or any combination thereof. It is also preferred to use oil from microbes of the genera *Mortierella, Pythium, Crypthecodinium,* and *Nitzschia* or any combination thereof. Particularly preferred species from these genera are *M. alpina, P. insidiosum, C. cohnii* and *N. alba*. This preferred embodiment would provide an acceptable alternative for vegetarians, including breast-feeding or pregnant vegetarian women.

If desired, fish oil can be blended, or mixed, with any combination of, or individual, microbial oil to produce a composition which, when subsequently added to infant formula will alter the PUFA content thereof in a desirable manner. Such a composition would not be suitable for a strict vegetarian intake. A preferred fish oil is specially processed Menhaden Oil (produced by Zapata Hayne, Inc.) which typically contains about 9% DHA. Of course, other fish oils also can be used.

When DHASCO is to be blended with ARASCO, and no other PUFA-containing oils are to be utilized, it is desirable to blend suffici nt amounts of the oils to provide from about 1 to about 5 parts DHA with from

about 2 to about 12 parts ARA. A most preferred ratio of DHA to ARA is 1:3 respectively.

As another example, Menhaden fish oil, as noted above, typically contains about 9% by weight DHA. ARASCO typically contains about 20 - 40% by weight ARA. DHASCO typically contains about 25 - 40% by weight DHA. It has been found that a blend of 1 part Menhaden oil containing about 9% by weight DHA with 10 parts ARASCO containing about 33% by weight ARA and 3 parts DHASCO containing about 35% by weight DHA, when added to infant formula, causes the infant formula to closely approximate the ARA and DHA content of human breast milk. Other ratios can be readily calculated.

In another embodiment of the present invention is disclosed a process for making a supplement for infant formula or baby food which entails blending a DHA-containing oil with a GLA-containing oil. It is to be understood that, in general, any combination of GLA-, EPA-, ARA- or DHA-containing oils, with or without fish oil, can be used. The source of the GLA can be a vegetable oil, such as primrose, black currant or borage oil, or a microbial oil such as the oil from *Mucor javonicus* or *Mortierella isabellina*, for example. Table 1 sets forth the GLA composition of such oils. In a preferred aspect of this embodiment, about 1 part of Menhaden oil containing about 9% DHA, about 4 parts of GLA-containing oil containing about 18% GLA from black currant seed, and about 1 part of DHASCO containing about 33% DHA are blended together. Other ratios can be selected as desired.

Table 1. Fatty acids of commercially available oils containing GLA (from Lawson and Hughes, 1988 and Suzuki, 1989)

Fatty acyl group	Relative % of total acyl groups in oil from: Mucor javanicus*	Mortierella isabellina**	Evening Primrose	Black-currant	Borage
14:0	1.0	0.7	-	-	-
14:1	0.1	-	-	-	-
16:0	18.6	27.2	5.9	6.9	10.7
16:1	1.0	0.9	-	-	-
18:0	7.1	5.7	1.8	1.3	3.0
18:1	39.9	43.9	7.5	10.8	15.4
18:2	8.9	12.0	74.8	46.7	38.1
γ-18:3(ω6)	17.9	8.3	9.3	15.9	24.8
α-18:3(ω3)	-	-	-	13.0	-
18:4(ω3)	-	-	-	2.9	-
20:0	-	0.6	-	-	-
20:1(ω9)	-	-	-	-	4.0
22.0	-	0.1	-	-	-
22:1(ω9)	-	0.2	-	-	2.2
24:0	0.6	-	-	-	-

* Produced by J. & E. Sturge Ltd., Selby, N. Yorks., U.K.
** Produced by Idemitzu Petro Chemical Co. Ltd., Tokyo, Japan.

Lawson - Lipids 23:313-317 (1988)
Suzuki - In Biotechnology for the Fats and Oils Industry p.110-116. Amer Oil Chem. Soc. Press (1989).

A composition including a blend of any combination of the above-described microbial oils with or without either, or both, fish oil and vegetable oil is another aspect of the present invention. While the composition includes any ratios of the oils, the ratios previously described are preferred.

In another preferred embodiment, the composition serves as a nutritional supplement. Typically, such supplements are encapsulated, such as in gelatin capsules. Such capsules provide an easy form of administration to persons having a need for supplementation, such as pregnant or nursing women. However, parenteral administration is a viable option and in one embodiment the composition comprises the fat component of a total parenteral nutritional formula. Such formulas are known and commercially available.

As will be understood, the composition of the present invention is particularly useful as a dietary supplement for pregnant or nursing women. Vegetarian women, in particular, may require increased amounts of DHA and ARA, yet have been precluded from obtaining such in the past because the only available sources were animal.

The invention having been previously described in general, reference is now had to the following non-limiting examples for illustrative purposes only.

Examples

Example 1. Preparation of *P. insidiosum* lipid

In an 80 liter (gross volume) fermentor, 51 liters of tap water, 1.2 kg glucose, 240 grams of yeast extract and 15 ml of MAZU 210S® antifoam were combined. The fermentor was sterilized at 121°C for 45 minutes. An additional 5 liters of condensate water were added

during the sterilization process. The pH was adjusted to 6.2, and approximately 1 liter of inoculum (at a cell density of 5-10g/l) of Pythium insidiosum (ATCC #28251) then was added. The agitation rate was adjusted to 125 RPM (250 cm/sec tip speed) and the aeration rate was set at 1 SCMF (standard cubic feet per minute). At hour 24 in the operation the aeration rate was increased to 3 SCFM. At hour 28 an additional 2 liters of 50% glucose syrup (1 kg glucose) were added. At hour 50 the fermentor was harvested, resulting in a yield of about 2.2 kg wet weight (approximately 15 g dry weight) per liter. Harvested biomass was squeezed to a high solids cake (50% solids) on a suction filter before freeze drying. The dried biomass was ground with a mortar and pestle and extracted with 1 liter of hexane per 200 grams of dry biomass at room temperature under continuous stirring for 2 hours. The mixture then was filtered and the filtrate evaporated to yield about 5-6 grams of crude oil per 100 grams of dry biomass. The biomass then was reextracted with 1 liter of ethanol per 20 grams of dry biomass for 1 hour at room temperature, filtered, and the solvent evaporated yielding an additional 22 grams of crude oil per 100 grams of dry biomass. The second fraction was predominantly phospholipids whereas the first fraction contained a mixture of phospholipids and triglycerides. The combined fractions produced an oil containing about 30-35% arachidonic acid and no detectable EPA.

Example 2. Preparation of *M. alpina* lipid

Mortierella alpina (ATCC #42430) was grown in a 2 liter shake flask containing 1 liter of tap water and

20 grams of potato dextrose medium. The flask was under constant orbital agitation and was maintained at 25°C for seven days. After harvesting by centrifugation, the biomass was freeze dried yielding about 8 grams of lipid-rich mycelia. The mycelia was extracted using hexane as in example #1 and about 2.4g of crude oil resulted. This oil contains about 23% arachidonic acid.

Example 3

Into a 30-liter working volume STF was loaded a medium of one quarter strength artificial seawater. Six liters of IO were combined with 18 liters of tap water. The fermentor containing the medium was sterilized and cooled to 28°C. Four hundred ml of concentrated YE (455g/l), 900 ml of glucose syrup (400 g/l) and one liter of inoculum from a seed fermentor containing about 2 x 10^7 *C. cohnii* cells/ml or a biomass of 20 g/liter (yielding a final concentration of about 10^5 cells/ml or a biomass of about 700 mg/liter), were added to the medium. The *C. cohnii* cells, designated MK8840, were obtained from the American Type Culture Collection as ATCC 40750. Agitation was set at 120 cm/sec tip speed and aeration was set at 1 VVM (30 liters per minute). Additional glucose syrup (900 ml) was added after 30 hours and another 4.2 liters over the next 42 hours. Thus 6 liters of glucose syrup were added in total. Concentrated YE solution (400 ml) was added at hour 6 and another 1.2 liters were added over the next 48 hours until a total of 2.0 liters had been added. To maintain the D.O. at greater than 20%, at 24 hours the agitation tip speed was increased to 150 cm/sec and at 48 hours to 160 cm/sec. At 72 hours, the tip sp ed was increased to 200 cm/sec and the culture

was p rmitted to grow for an additional time sufficient to convert the final charge of glucose into cellular oil. The culture was then harvested by centrifugation with the cell pellet retained. The harvested pellet of cells was frozen and dried (lyophilized) to about a 4% moisture content. Hexane (2.8 liters) was added to the dried biomass and stirred in a glass kettle for 1.5 hours at 50°C. A rotary evaporator was used to remove the hexane, producing about 175 g of crude DHA-containing oil.

Example 4

Into a conventional 30 liter stirred tank fermentor (STF) is added the nutrient medium of Table A, exclusive of the vitamins, glucose and silicate. The fermentor is equipped with a Rushton-type turbine agitator. The STF and the medium are sterilized. After cooling the medium to about 30°C, the vitamins are added, followed by the addition of sufficient amounts of 40% glucose syrup to provide a glucose concentration of about 80 g/l. Concentrated sodium metasilicate pentahydrate (100 g/l) is then added to provide a total silicate concentration of about 200 mg/l. Next, the inoculating amount of culture of *N. alba* cells obtained from the American Type Culture Collection as ATCC 40775, is added in an amount approximately equal to 5% of the total volume of the fermentor, e.g. 1.5 liters/30 liters. Agitation is commenced with the tip speed set to 85-90 cm/sec and air sparging at 1 VVM started. Over about 16 hours an additional charge of concentrated metasilicate (0.53 liters) is added and the agitation speed increased to 126 cm/sec. Over about the next 24 hours, more concentrated silicate (0.83 liters) is added.

Agitation speed again is increased to about 180-185 cm/sec. Over about the next 3 hours an additional 0.15 liters of concentrated metasilicate is added. Thus, the total amount of metasilicate added is about 156 grams or about 1.6 liters of concentrated solution. At about 48 hours additional glucose (about 5 liters) is added, for a total glucose addition of about 4.8 Kg or about 12 liters of 40% glucose syrup. The culture is permitted to grow for an additional 16 hours, maintaining the agitation speed and aeration rate. Then, the fermentor is harvested using a Sharples continuous flow centrifuge producing a biomass density of approximately 45-48 grams dry weight per liter. The resulting pellet, about 20-38% solids, is removed and frozen to about -20°C. A vacuum tray drier is used to remove water from the pellet. The single cell oil pellet then is extracted with hexane. The hexane subsequently is removed by distillation leaving the extracted single cell oil.

Table A

GROWTH MEDIUM COMPOSITION

Ingredients needed for 2x30L Fermentors and 2x350L Fermentors.

Recipe		Total 30L-Batch	Total 350L-Batch
19g/L	I.O. (Instant Ocean®)	570g	6.65Kg
3g/L	$NaNO_3$	90g	1.05Kg
0.5g/L	$NaH_2PO_4 \cdot H_2O$	15g	175g
0.2g/L	$Na_2SiO_3 \cdot 5H_2O$	6g	70g
6ml/L	f/2 TM (trace metals)	180ml	2.1L
60mg/L	H_3BO_3	1.8g	21g
6mg/L	Na_2SeO_3	180mg	2.1g
10mg/L	NaF	300mg	3.5g
40mg/L	$SrCl_2 \cdot 6H_2O$	1.2g	14g
150mg/L	KBr	4.5g	52.5g
0.5g/L	KCl	15g	175g
2ml/L	B_6 TM (trace metals)	60ml	700ml
After Sterilization			
0.1ml/L of	0.1mg/ml B_{12}	3ml	35ml
0.1ml/L of	0.1mg/ml Biotin	3ml	35ml
2ml/L of	1mg/ml Thiamine HCl	60ml	700ml
Glucose:	(1) Start with 80g/L (40% stock solution)	6L	70L
	(2) Add another 40g/l 1 and 2 (additional 6 liters on day 2)	3l	35L
Silicate:	Add 60ml/liter of 100g/liter stock solution add additional amounts of stock solution over 48 hours	1.8L	21L

Example 5. Preparation of Oil Mix #1 and addition to infant formula.

The first mixture represents a totally vegetarian source of an arachidonic and docosahexaenoic acid supplement. This supplement would be considered acceptable to persons restricted to a vegetarian diet. Sanders et al. (Amer. J. Clin. Nutr. 31:805; 1978) have reported that the DHA levels in the breast milk of vegetarian mothers are depressed. Enteral supplementation of a blend of DHA single cell oil and ARA single cell oil will elevate the serum and, hence, breast milk levels of DHA to that of omnivorous mothers. This blend is prepared by mixing one part DHASCO containing about 35% DHA (obtained from *Crypthecodinium cohnii* as described in Example 3) with three parts ARASCO containing about 33% ARA (obtained from *Pythium insidiosum* as described in Example 1). The resulting mixture, or blend, has the fatty acid composition shown in Table 2. The blend is mixed in a ratio of one part blend to forty parts of the oils regularly in infant formula, typically about 2.8 - 3.0 grams per 100 ml of formula. At a normal fat content of 30g fat per liter of Similac® infant formula, this corresponds to the addition of 750 mg per liter of prepared formula. This supplement provides ARA and DHA levels equivalent to human breast milk.

Table 2. Composition of a blend of DHA oil and ARA oil in proportions of 1:3 by weight.

Fatty Acid	oil mix #1	Infant formula	formula + mix #1	breast milk
8:0 + 10:0	0.00	41.8	40.78	1.74
12:0 + 14:0	13.63	20.7	20.53	14.95
16:0	17.05	6.8	7.05	19.82
16:1	7.88	0.2	0.39	3.20
18:0	0.00	2.3	2.24	5.91
18:1	7.48	10.0	9.94	34.82
18:2 n6	7.20	17.4	17.15	16.00
18:3 n3	2.25	0.9	0.93	0.62
18:3 n6	4.50	--	0.11	0.00
20:1	--	0.1	0.10	1.10
20:2 n6	--	--	0.00	0.61
20:3 n6	--	--	0.00	0.42
20:4 n6	24.75	--	0.60	0.59
20:5 n3	--	--	0.00	0.03
22:1	--	--	0.00	0.10
22:4 n6	--	--	0.00	0.21
22:5 n6	--	--	0.00	0.22
22:6 n3	8.98	--	0.22	0.19

Example 6. Preparation of Oil Mix #2 and addition to infant formula.

This mixture represents a totally vegetarian source of long chain PUFAs and would be considered acceptable to persons restricted to a vegetarian diet. This blend is prepared by mixing three parts DHASCO containing about 35% DHA (obtained from *Crypthecodinium cohnii* as described in Example 3) with ten parts ARASCO containing about 33% ARA (obtained from *Pythium insidiosum* as described in Example 1) and five parts EPASO containing about 5% EPA (obtained from *N. alba* as described in Example 4). The resulting mixture, or blend, has the fatty acid composition shown in Table 3. The blend is mixed in a ratio of one part blend to thirty parts of the oils regularly in infant formula. At a normal fat content of 30g fat per liter of Similac® infant formula, this would correspond to the addition of one gram per liter of prepared formula. This supplement provides ARA, DHA and EPA levels equivalent to human breast milk.

Table 3. Composition of a blend of DHA oil, ARA oil and EPA oil in proportions of 3:10:5 by weight.

Fatty Acid	oil mix #2	Infant formula	formula + mix #2	breast milk
8:0 + 10:0	0.00	41.8	40.45	1.74
12:0 + 14:0	16.64	20.7	20.57	14.95
16:0	21.61	6.8	7.28	19.82
16:1	6.55	0.2	0.40	3.20
18:0	0.28	2.3	2.23	5.91
18:1	12.91	10.0	10.09	34.82
18:2 n6	5.87	17.4	17.03	16.00
18:3 n3	1.88	0.9	0.93	0.62
18:3 n6	3.48	--	0.11	0.00
20:1	--	0.1	0.10	1.10
20:2 n6	--	--	0.00	0.61
20:3 n6	0.19	--	0.01	0.42
20:4 n6	18.52	--	0.60	0.59
20:5 n3	0.76	--	0.02	0.03
22:1	--	--	0.00	0.10
22:4 n6	0.11	--	0.00	0.21
22:5 n6	--	--	0.00	0.22
22:6 n3	6.24	--	0.20	0.19

Example 7. Preparation of Oil Mix #3 and addition to infant formula.

This mixture is a blend of ARASCO with fish oils. Oil mixture #3 is prepared by adding one part specially processed Menhaden Oil (Zapata Hayne Inc.) containing about 9% DHA to one part of ARASCO, obtained from *Pythium insidiosum* as described previously containing about 33% ARA. The resultant fatty acid composition is shown in Table 4. This blend is mixed in a ratio of one part blend to thirty parts of the oils regularly in infant formula. At a normal fat content of 30 g fat per liter of infant formula, this corresponds to the addition of 1 gram per liter of prepared formula. This supplement provides ARA and DHA levels equivalent to human breast milk, but the EPA levels are about eight-fold higher than those in breast milk.

Table 4. Composition of a blend of SPMO* and ARA oil in proportions of 1:1 by weight.

Fatty Acid	oil mix #3	Infant formula	formula + mix #3	breast milk
8:0 + 10:0	0.0	41.8	40.45	1.74
12:0 + 14:0	10.2	20.7	20.36	14.95
16:0	15.5	6.8	7.08	19.80
16:1	11.5	0.2	0.56	3.20
18:0	1.41	2.3	2.27	5.91
18:1	8.79	10.0	9.96	34.82
18:2 n6	5.57	17.4	17.02	16.00
18:3 n3	2.31	0.9	0.95	0.62
18:3 n6	3.00	--	0.10	0.00
20:1	0.78	0.1	0.12	1.10
20:2 n6	0.00	--	0.00	0.61
20:3 n6	0.00	--	0.00	0.42
20:4 n6	17.52	--	0.57	0.59
20:5 n3	7.76	--	0.25	0.03
22:1	0.00	--	0.00	0.10
22:4 n6	0.00	--	0.00	0.21
22:5 n6	1.21	--	0.04	0.22
22:6 n3	4.57	--	0.15	0.19

* Specially Processed Menhaden Oil.

Example 8. Preparation of Oil Mix #4 and addition to infant formula

Oil mixture #4 was developed to utilize GLA in place of arachidonic acid. This blend was prepared by mixing one part specially prepared Menhaden oil containing about 9% DHA (Zapata Hayne Inc.) with four parts black currant seed oil containing about 18% GLA and one part DHASCO containing about 35% DHA. The resultant fatty acid composition is shown in Table 5. This blend is mixed in a ratio of one part blend to forty parts of the oils regularly in infant formula. At a normal fat content of 30g fat per liter, this would correspond to the addition of 750 mg per liter of prepared formula. This supplement provides EPA and DHA levels equivalent to human breast milk. The ARA levels are about one tenth the level in human breast milk. However, the GLA levels are twenty to fifty times higher than the GLA levels in breast milk which typically are minute.

Table 5. Composition of a blend of SPMO, BCO and DHA oil in proportions of 1:4:1 by weight.

Fatty Acid	oil mix #4	Infant formula	formula + mix #4	breast milk
8:0 + 10:0	0.0	41.8	40.78	1.74
12:0 + 14:0	4.83	20.7	20.31	14.95
16:0	11.86	6.8	6.92	19.80
16:1	2.09	0.2	0.25	3.20
18:0	1.34	2.3	2.28	5.91
18:1	10.98	10.0	10.02	34.82
18:2 n6	31.39	17.4	17.74	16.00
18:3 n3	8.94	0.9	1.10	0.62
18:3 n6	10.60	--	0.26	0.00
20:1	0.26	0.1	0.10	1.10
20:2 n6	--	--	0.00	0.61
20:3 n6	--	--	0.00	0.42
20:4 n6	0.34	--	0.06	0.59
20:5 n3	2.59	--	0.00	0.03
22:1	--	--	0.00	0.10
22:4 n6	--	--	0.00	0.21
22:5 n6	0.40	--	0.01	0.22
22:6 n3	7.51	--	0.18	0.19

Example 9. Preparation of Oil Mix #5 and addition to infant formula

Oil mixture #5 was developed to best approximate the composition of DHA, ARA and EPA of human breast milk. This oil blend was prepared by mixing one part specially prepared Menhaden oil containing about 9% DHA (Zapata Hayne Inc.) with ten parts of ARASCO containing about 33% ARA and three parts DHASCO containing about 35% DHA. The resultant fatty acid composition is shown in Table 6. This blend is mixed in a ratio of one part blend to forty parts of the oils regularly in infant formula. At a normal fat content of 30g fat per liter of infant formula, this corresponds to the addition of 750 mg per liter of prepared formula. This supplement provides EPA, DHA and ARA levels substantially equivalent to those levels in human breast milk.

Table 6. Composition of a blend of SPMO, ARA oil and DHA oil in proportions of 1:10:3 by weight.

Fatty Acid	oil mix #5	Infant formula	formula + mix #5	breast milk
8:0 + 10:0	0.00	41.8	40.78	1.74
12:0 + 14:0	13.14	20.7	20.52	14.95
16:0	16.83	6.8	7.04	19.80
16:1	8.39	0.2	0.40	3.20
18:0	0.20	2.3	2.25	5.91
18:1	7.66	10.0	9.94	34.82
18:2 n6	6.97	17.4	17.15	16.00
18:3 n3	2.26	0.9	0.93	0.62
18:3 n6	0.25	--	0.01	0.00
20:1	0.11	0.1	0.10	1.10
20:2 n6	--	--	0.00	0.61
20:3 n6	--	--	0.00	0.42
20:4 n6	23.72	--	0.58	0.59
20:5 n3	1.11	--	0.03	0.03
22:1	--	--	0.00	0.10
22:4 n6	--	--	0.00	0.21
22:5 n6	0.17	--	0.00	0.22
22:6 n3	8.35	--	0.20	0.19

Example 10. Preparation of Oil MIx #6 and addition to infant formula

This mixture represents a totally vegetarian source of arachidonic and docosahexaenoic acid. This supplement would be considered acceptable to persons restricted to a vegetarian diet. Sanders et al. (*American J. Clin. Nutr. 31*:805; 1978) have reported that the DHA levels in the breast milk of vegetarian mothers are depressed. Enteral supplementation of the blend will elevate the serum and hence breast milk levels of DHA to that of omnivorous mothers. This blend is prepared by mixing one part DHA oil (obtained from *Crypthecodinium cohnii* as described in Example 3) with five parts ARA oil (obtained from *Mortierella alpina* as described in Example 2). The resulting mixture has the fatty acid composition shown in Table 7. This blend is mixed in a ratio of one part to thirty-five parts of the oils regularly in infant formula. At a normal fat content of 35 g fat per liter, this would correspond to the addition of 1 g per liter of prepared formula. This supplement provides ARA and DHA levels equivalent to human breast milk.

Table 7. Composition of a blend of DHA oil and ARA oil in proportions of 1:5 by weight.

Fatty Acid	Oil Mix #6	Infant Formula	Formula + Mix #6	Breast Milk
8:0 + 10:0	0.0	41.8	40.6	1.74
12:0 + 14:0	4.0	20.7	20.2	14.95
16:0	16.8	6.8	7.1	19.82
16:1	0.5	0.2	0.2	3.2
18:0	11.0	2.3	2.5	5.91
18:1	17.8	10.0	10.0	34.82
18:2 n6	11.1	17.4	17.2	16.00
18:3 n3	5.2	0.9	0.9	0.62
18:3 n6	4.5	---	0.1	0.00
20:1	---	0.1	0.1	1.10
20:2 n6	---	---	0.0	0.61
20:3 n6	6.0	---	0.17	0.42
20:4 n6	20.1	---	0.57	0.59
20:5 n3	0.1	---	0.01	0.03
22:1	---	---	0.00	0.10
22:4 n6	2.0	---	0.06	0.21
22:5 n6	---	---	0.00	0.22
22:6 n3	6.1	---	0.18	0.19

We claim:

1. A process for supplementing infant formula comprising:

(a) obtaining at least two different long chain polyunsaturated fatty acid-containing microbial oils from at least two different microbial sources, and

(b) adding said oils to said infant formula.

2. The process of claim 1, wherein said oils are selected from omega-3 and omega-6 fatty acids.

3. A process for altering the composition of infant formula, such that the relative amount of omega-3 and omega-6 polyunsaturated fatty acids in said formula is substantially similar to the amount of said polyunsaturated fatty acids contained in human breast milk which comprises:

(a) obtaining microbial oils containing said polyunsaturated fatty acids from at least two species of microbes,

(b) blending said oils together, and

(c) adding said blend of oils to said infant formula in an amount effective to provide said formula with amounts of said polyunsaturated fatty acids substantially similar to the amounts in human breast milk.

4. The process of claim 3, wherein said oils are ARASCO and DHASCO.

5. The process of claim 4, wherein said DHASCO is blended with said ARASCO in a ratio of from about 1 to about 5 parts DHASCO to about 2 to about 12 parts ARASCO by weight of said blend.

6. The process of claim 5, wherein the ratio of DHASCO to ARASCO comprises about 1:3.

7. The process of claim 4, wherein said genera are selected from fungi and microalgae.

8. The process of claim 7, wherein said fungi comprise *Pythium* or *Mortierella*.

9. The process of claim 8, wherein said microalgae comprise *Crypthecodinium sp.*

10. The process of claim 7, wherein said fungi comprises *Pythium* or *Mortierella* and said microalgae comprises *Crypthecodinium*.

11. The process of claim 10, wherein said *Pythium* comprises *P. insidiosum*, said *Mortierella* comprises *M. alpina* and said *Crypthecodinium* comprises *C. cohnii*.

12. The process of claim 4, wherein said oil further comprises EPASCO.

13. The process of claim 12, wherein said genera are selected from fungi and microalgae.

14. The process of claim 13, wherein said fungi comprise *Pythium* or *Mortierella*.

15. The process of claim 13, wherein said microalgae comprise *Crypthecodinium* and *Nitzschia*.

16. The process of claim 13, wherein said fungi comprise *Pythium* and *Mortierella* and said microalgae comprises *Crypthecodinium* and *Nitzschia sp.*

17. The process of claim 16, wherein said *Pythium* comprises *P. insidiosum* and said *Crypthecodium* comprises *C. cohnii* and said *Nitzschia* comprises *N. alba* and said *Mortierella* comprises *M. alpina*.

18. The process of claim 3, further comprising blending said microbial oils with fish oil prior to adding said blend to said infant formula.

19. The process of claim 18, wherein said fish oil comprises about 1 part and said microbial oils comprise from about 1 to about 15 parts by weight of said blend.

20. Th process of claim 19, wherein said microbial oils are s lected from DHASCO and ARASCO and

the ratio of said oils comprises about one part fish oil to ten parts ARASCO to three parts DHASCO.

21. A process for making a supplement for infant formula, comprising:

(a) obtaining a DHA-containing microbial oil and

(b) blending said oil with a gamma linolenic acid-containing oil, thereby producing said supplement.

22. The process of claim 21, wherein said linolenic acid-containing oil comprises primrose, borage, or black currant seed oil.

23. The process of claim 21, wherein said linolenic acid containing-oil comprises a microbial oil.

24. The process of claim 23, further comprising obtaining said linolenic acid-containing oil from *Mucor javonicus* or *Mortierella isabellina*.

25. The process of claim 21, further comprising blending with said DHA-containing microbial oil and said linolenic acid-containing oil an EPA-containing oil.

26. The process of claim 25, wherein said EPA-containing oil comprises fish oil.

27. The process of claim 26, wherein said fish oil comprises about one part, said linolenic acid-containing oil comprises about 4 parts and said DHA-containing oil comprises about 1 part by weight of said blend.

28. The process of claim 26, wherein said fish oil comprises about one part, said linolenic acid-containing oil comprises about 4 parts and said DHA-containing oil comprises about 1 part by weight of said blend.

29. A composition comprising a blend of at least two long-chain polyunsaturated fatty acid-containing microbial oils.

30. The composition of claim 29, wherein said oils are selected from ARASCO and DHASCO.

31. The composition of claim 30, wherein said DHASCO is blended with said ARASCO such that a ratio of from about 1 to about 5 parts DHA and about 2 to about 12 parts ARA by weight of said blend is obtained.

32. The composition of claim 31, wherein the ratio of DHA to ARA comprises about 1:3 respectively.

33. The composition of claim 30, wherein said oil further comprises EPASCO.

34. The composition of claim 29, wherein said composition further comprises fish oil.

35. The composition of claim 34, wherein said fish oil comprises about 1 part and said microbial oils comprise from about 1 to about 15 parts by weight of said blend.

36. A composition comprising a blend of a DHA-containing microbial oil and a gamma linolenic acid-containing oil.

37. The composition of claim 36, wherein said linolenic acid-containing oil comprises primrose, borage, or black currant seed oil.

38. The composition of claim 36, wherein said linolenic acid containing-oil is an oil obtained from a microbe.

39. The composition of claim 38, wherein said microbe comprises *Mucor javonicus* or *Mortierella isabellina*.

40. The composition of claim 36, further comprising an EPA-containing oil.

41. The composition of claim 40, wherein said EPA-containing oil comprises fish oil.

42. The composition of claim 41, wherein said fish oil comprises about one part, said linolenic acid-containing oil comprises about 4 parts and said DHA-containing oil comprises about 1 part by weight of said blend.

43. Nutritional supplements comprising mixtures of polyunsaturated fatty acid-containing microbial oil.

44. Nutritional supplements comprising mixtures of DHASCO and a gamma linolenic acid-containing oil.

45. Nutritional supplements comprising mixtures of ARASCO and fish oils.

46. The supplement of claim 43, further comprising fish oil.

47. The supplement of claim 46, wherein said fish oil comprises about 1 part and said microbial oils comprise from about 1 to about 15 parts by weight of said supplement.

48. The supplement of claim 47, wherein said microbial oils comprise DHASCO and ARASCO and the ratio of said oils comprises about one part fish oil to ten parts ARASCO to three parts DHASCO.

49. The supplement of claim 43, wherein said mixture comprises ARASCO and DHASCO.

50. The supplement of claim 49, wherein said DHASCO is blended with said ARASCO in a ratio of from about 1 to about 5 parts DHA to about 2 to about 12 parts ARA by weight of said supplement.

51. The supplement of claim 50, wherein the ratio of DHA to ARA comprises about 1:3.

52. The supplement of claim 44, wherein said linolenic acid-containing oil comprises primrose, borage, or black currant seed oil.

53. The supplement of claim 44, wherein said linolenic acid containing-oil is obtained from a microbe.

54. The supplement of claim 53, wherein said microbe comprises *Mucor javonicus* or *Mortierella isabellina*.

55. The supplement of claim 44, further comprising an EPA-containing oil.

56. The supplement of claim 55, wherein said EPA-containing oil comprises fish oil.

57. The supplement of claim 56, wherein said fish oil comprises about one part, said linolenic acid-containing oil comprises about 4 parts and said DHA-containing oil comprises about 1 part by weight of said blend.

58. The supplement of claim 43, wherein said supplement is a human nutritional supplement.

59. The supplement of claim 58, wherein said human is a baby.

60. The supplement of claim 58, wherein said human is a pregnant or nursing woman.

61. The composition of claim 29, wherein said composition comprises an additive for supplementing an infant formula.

62. The composition of claim 29, wherein said composition comprises a total parenteral nutritional formula.

63. The composition of claim 34, said composition comprising an additive for supplementing an infant formula.

64. The composition of claim 34, wherein said composition comprises a total parenteral nutritional formula.

65. The composition of claim 36, wherein said composition comprises an additive for supplementing an infant formula.

66. The composition of claim 36, wherein said composition comprises a total parenteral nutritional formula.

67. The composition of claim 45, wherein said composition comprises an additive for supplementing an infant formula.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00522

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 31/20
US CL : 514/560

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	514/560

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A, 0,269,351 (LION CORPORATION) 01 June 1988, See entire document.	1-28
Y	Abstract of JP 1,132,371 (HANADA ET AL.) 24 May 1989, "Novel Microorganism and Production of Lipid Component of High Content of Gamma-Linolenic Acid."	1-28
Y	US, A, 4,670,285 (CLANDInin ET AL.) 02 June 1987, See column 2, line 61 - column 3, line 8 and column 6, line 8 - column 7, line 17. . .	29-67
Y	US, A, 4,938,984 (TRAITLER ET AL.) 03 July 1990, See column 3, lines 21-61.	29-67

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 June 1992	27 JUL 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Kimberly Jordan

FURTHER INFORMATI N CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... . because they relate to subject matter[12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out[13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

Group I: Claims 1-28 drawn to processes for supplementing infant formula with long chain polyunsaturated fatty acid containing microbial oils.
Group II: Claims 29-67 drawn compositions containing long chain polyunsaturated fatty acid containing microbial oils.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.